Annals of Botany **102**: 979–987, 2008
doi:10.1093/aob/mcn189, available online at www.aob.oxfordjournals.org

OXFORD JOURNALS
OXFORD UNIVERSITY PRESS

Plant Enemy-derived Elicitors Increase the Foliar Tannin Concentration of *Onobrychis viciifolia* Without a Trade-off to Growth

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Received: 23 June 2008 Returned for revision: 18 August 2008 Accepted: 4 September 2008 Published electronically: 9 October 2008

• *Background and Aims* Molecular experiments suggest that the regulation of the biosynthesis of condensed tannin (CT) is sensitive to the presence of plant enemies. The enemy-specific response of CT concentrations to simulated attacks by pathogenic fungi, bacteria or herbivores was studied in *Onobrychis viciifolia* grown at four levels of nutrient availability. It was hypothesized that CT concentrations increase in response to an attack, and that constitutive and induced levels of CT are higher at low than at high nutrient availability. Investment in CT was also predicted to be negatively related to plant growth.
• *Methods* Recently discovered substances by which plants recognize their opponents (i.e. elicitors) were used to simulate attacks to *Onobrychis viciifolia* grown at 0·0027, 0·075, 0·67 or 2 mM phosphorus in the nutrient solution.
• *Key Results* Relative growth rate and final biomass ($P < 0{\cdot}001$) were highest at 0·67 mM of phosphorus. CT concentrations decreased with increasing phosphorus availability, from 94·9 to 69·0 mg g^{-1} leaf dry weight ($P < 0{\cdot}001$). Compared with unscathed plants, sterile mere mechanical wounding reduced tannin concentrations from 83·8 to 69·3 mg g^{-1} leaf dry weight ($P < 0{\cdot}01$). Local CT concentrations were higher when wounded leaves were additionally treated with fungal (+15·9 %), bacterial (+19·6 %) or insect (+31·0 %) elicitors (each elicitor; $P < 0{\cdot}05$); however, only the insect elicitor (saliva of the lepidopteron *Spodoptera littoralis*) induced CT concentrations higher than those of unscathed leaves.
• *Conclusions* CT concentrations were inducible in the vicinity of the wound but the level of induction was unrelated to the nutrient status of the plant. There was no evidence of a growth-defence trade-off. The inverse relathionship between CT concentrations and nutrient availability appears to reflect passive growth dilution at high nutrient availability, rather than surplus CT production at low nutrient availability.

Key words: *Onobrychis viciifolia*, condensed tannin, elicitor, plant–herbivore interaction, plant–pathogen interaction, growth–defence trade-off, *Spodoptera littoralis*, volicitin, Pen, chitin, elf18, flg22.

INTRODUCTION

Although the primary function of condensed tannins (CTs) in terms of evolutionary processes is disputable (Edwards, 1992), they are usually considered an important aspect of chemical plant defence. CTs can have antibiotic activity (Brownlee *et al.*, 1990; Heil *et al.*, 2002) and deter herbivores (Bernays, 1981; Coley, 1986; Bialczyk *et al.*, 1999). Moreover, various studies have shown that the abundance and species richness of leaf-eating insects tend to be negatively correlated with foliar CT concentrations (Feeny, 1976; Coley, 1986; Bialczyk *et al.*, 1999; Forkner *et al.*, 2004).

There is molecular evidence that the regulation of the biosynthetic pathway of CTs is closely linked to the plant's enemy detection system (Groten and Barz, 2000; Richard *et al.*, 2000; Peters and Constabel, 2002; Rossi *et al.*, 2004; Ralph *et al.*, 2006, Farag *et al.*, 2008). This finding challenges the classical view of CTs as constitutive, non-inducible plant defences. Key enzymes such as the dihydroflavonol reductase are inducible by the real or simulated presence of herbivores or pathogens, and in cell-suspension cultures the reaction time and intensity were found to be enemy specific (Groten and Barz, 2000, Farag *et al.*, 2008).

The aim of the present experiment was to investigate the response of foliar tannin concentrations to simulated attacks by pathogenic fungi, bacteria and herbivores, and to study the potential interaction of these responses with the nutrient status of the plant (source–sink balance). The recent identification of substances (elicitors) by which plants specifically recognize their enemies allowed attacks by natural plant enemies to be simulated, while making only small, standardized wounds that scarcely interfered with carbon assimilation. The surveillance system of plants is very sensitive and includes chemoreceptors for elicitors that are characteristic of entire groups or classes of organisms, and also receptors and mechanisms for the recognition of particular strains within these groups (Salzer *et al.*, 2000). In this experiment, the aim was to determine how the CT concentration of *Onobrychis*, as a model plant, responds to attacks by fungi, bacteria and insects in general, rather than to attempt to understand the plant response to particular elicitors in detail. Therefore elicitor mixtures were designed to represent these three biotic groups; where possible, elicitors were chosen that are common to many species within a group and for which an activation of plant defence responses

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has been demonstrated in at least one but in most cases several only distantly related plant model systems.

It was expected that a higher concentration of CT would be found in leaves that were wounded than in unscathed leaves, especially when elicitors were applied to the wound. It was also expected that plants grown under low nutrient conditions would have a higher constitutive level of CT and a stronger potential for induction than those grown under nutrient-rich conditions. One can arrive at these hypotheses by either thinking about the production costs of tissues in terms of resources or by weighing the severity of a loss of tissue against the investment in chemical plant defences that is needed to protect it (Coley *et al.*, 1985; Craine *et al.*, 2003; Stamp, 2003). Finally, the experimental design – with four levels of nutrient availability – made it possible to find out if there is a trade-off in the resources required for growth and CT production by testing the central but rarely addressed sub-hypothesis that, for a given level of nutrient availability, the correlation between growth (i.e. increase in biomass) and the concentration of CTs should be more negative at moderate and high nutrient availability than at low nutrient availability (Herms and Mattson, 1992; Stamp, 2003).

MATERIALS AND METHODS

Experimental design and plant material

In a greenhouse experiment, 120 plants of *Onobrychis viciifolia* ('Visnovsky'; Fabaceae) were arranged in a split-plot design with six blocks (i.e. replicates) and four levels of phosphorus availability as the main-plot factor, and five elicitor treatments (including the two control treatments) as the sub-plot factor. Each main plot consisted of a container (volume 24 L; surface 920 cm^2) which was filled with quartz sand (0·7–1·2 mm) and contained five experimental and five additional non-experimental plants. The experiment involved three phases: (1) an establishment period of 2 months when all plants were grown under the same conditions; (2) a period of 1 month when plants were grown with one of four nutrient solutions differing in phosphorus concentration; and (3) the treatment phase, in which plants were either left undamaged, or were wounded, or wounded and additionally treated with one of three possible elicitor mixtures.

Phase 1: establishment of experimental plants and nodulation. During the establishment period, plants were irrigated with 800 mL $container^{-1}$ d^{-1} of a full nutrient solution (Hammer *et al.*, 1978), but with reduced concentrations of phosphate (0·075 mM KH_2PO_4) and nitrogen (1·5 mM), as was also done by Almeida *et al.* (1999). Between September and December, normal daylight was supplemented with artificial light from 0600 h to 2000 h. The day/night temperatures were 22/15 °C and relative air humidity was 60/90 %. To ensure that nodulation occurred in the artificial substrate, plants were inoculated with *Rhizobium* sp. cultures derived from field-grown plants of the same plant species and cultivar (Vincent, 1970). By the end of the establishment period, the plants were 12·66 ± 0·40 cm tall, with an average biomass of 512 ± 31 mg dry weight (d. wt) and an average concentration of CT in their leaflets of 76·3 ± 3·2 mg CT g^{-1} d. wt.

Phase 2: nutrient treatments. In phase 2, the uniform nutrient solution was replaced by four solutions with 0·0027, 0·075, 0·67 and 2 mM KH_2PO_4 (Almeida *et al.*, 1999). The nitrogen concentration was maintained at 1·5 mM in all solutions, and all other nutrients were applied in the same concentrations as in the full nutrient solution (Hammer *et al.*, 1978). Based on the results of Almeida *et al.* (1999) with *Trifolium repens*, the experimental phosphorus concentrations were expected to impose a serious and a moderate limitation on plant growth at the lowest two levels, the third level was expected to provide optimal conditions for growth, while the highest level was intended to provide an excess of phosphorus. Daily irrigation volumes and other growth conditions remained constant as in phase one.

Phase 3: wounding and elicitor treatments. Plants were wounded twice, 9 d and 2 d prior to harvest, and treated with an elicitor when appropriate. Wounding was achieved by squeezing leaflets with sterile, grooved tweezers that left perforated marks of approx. 3·5 mm^2 $leaflet^{-1}$. On each of the plants to be wounded, three leaves were marked with a ribbon, and 15 leaflets from these leaves were damaged on two occasions (Fig. 1). For plants assigned to an elicitor treatment, 3 μL of the appropriate elicitor was applied to each wounded leaflet. At the given high concentration of elicitor (see below), this dose was believed to ensure a plant reaction in case the plant is sensitive to the elicitor.

There were two control and three elicitor treatments. The control treatments included completely unscathed plants and plants that were wounded but not treated with an elicitor. The 'fungal elicitor' was an aqueous solution

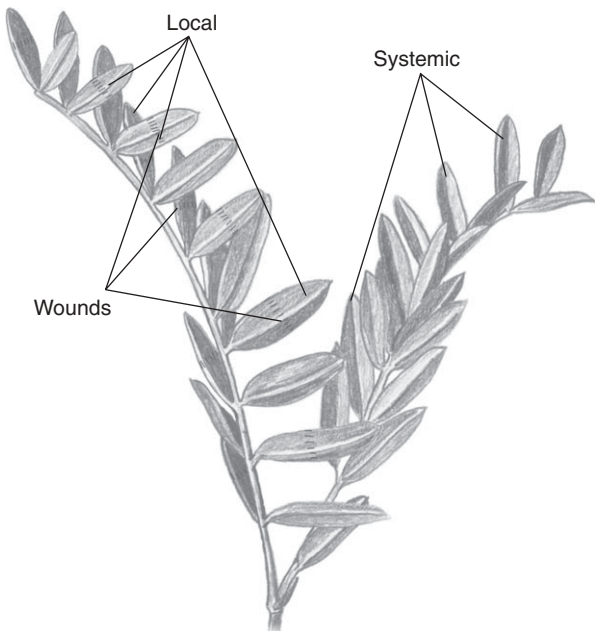

FIG. 1. Leaves of *Onobrychis viciifolia*. Local induction was measured in wounded leaflets. Systemic induction was measured in similar leaflets of an unwounded neighbouring leaf.

containing 100 μg mL^{-1} of hydrolysed chitin (Salzer *et al.*, 1997) and 100 μg mL^{-1} Pen (Thuerig *et al.*, 2006). The polysaccharide chitin is an important component of the cell walls of all true fungi, and triggers defence reactions in species as unrelated as *Solanum lycopersicum* (= *Lycopersicon esculentum*; Solanaceae; Felix *et al.*, 1999), *Arabidopsis thaliana* (Brassicaceae; Felix *et al.*, 1999) and *Picea abies* (Pinaceae; Salzer *et al.*, 1997). Pen was discovered recently in an aqueous extract of *Penicillium chrysogenum*, and as far as is known has not yet been chemically identified (Thuerig *et al.*, 2006). It is known to be recognized by tomato, tobacco and rice. In arabidopsis, Pen was shown to provide resistance against several pathogens without having a direct antimicrobial effect.

The 'bacterial elicitor' contained 50 μg mL^{-1} lyophilized *Micrococcus lysodeikticus* (Sigma; Felix and Boller, 2003) suspended in an aqueous solution of 1 μM flg22 (Felix *et al.*, 1999) and 1 μM elf18 (Kunze *et al.*, 2004; Zipfel *et al.*, 2006). Lyophilized *Micrococcus lysodeikticus* has elicitor activity due to the presence of cold-shock proteins (Felix and Boller, 2003). The peptides flg22 and elf18 are the most conserved domains of the bacterial flagellum protein 'flagellin' and of a prokaryotic elongation factor (i.e. EF-Tu), respectively. Both are very potent elicitors (Felix *et al.*, 1999; Kunze *et al.*, 2004; Zipfel *et al.*, 2006).

The 'insectan elicitor' consisted of freshly collected, undissolved and untreated regurgitant of *Spodoptera littoralis* caterpillars (Hoballah *et al.*, 2002) that had been previously raised on *Onobrychis viciifolia* plants. This spit contains 'volicitin' [*N*-(17-hydroxylinolenoyl)-L-glutamine] which is recognized in *Zea mays* (Poaceae) and *Vigna unguiculata* (Fabaceae), and triggers the release of a blend of small, volatile terpenes that attract parasitoid wasps of the herbivore.

Harvest and chemical analyses

Wounded leaflets (local) and comparable leaflets on unwounded leaves of the same plant (systemic) were collected and frozen (Fig. 1). This material was then lyophilized and ground to a fine powder using a ball mill. Roots were washed and dried with the other remaining tissues at 80 °C to determine the total biomass of each plant.

CTs were quantified photometrically in a butanol–hydrochloric acid (BuOH–HCl) assay adapted from Terrill *et al.* (1992). Approximately 50 mg plant powder was extracted three times in Teflon tubes using 5 mL of a 7 : 3 (v/v) acetone/water solution with 1 g L^{-1} ascorbic acid mixed with 4 mL diethyl ether. After each extraction, the tubes were centrifuged and the supernatants combined. The upper phase containing lipids and other non-polar molecules was discarded and the lower aqueous phase containing tannins was concentrated by rotary evaporation at 40 °C and 400 mbar. The resulting aqueous solution was made up to 20 mL with distilled water and the solid residue was stored at 4 °C for later use. A 1-mL aliquot of the aqueous solution was added to 6 mL of a freshly prepared BuOH–HCl solution (950 mL BuOH and 50 ml HCl, 37 %) and heated under reflux at 95 °C for 75 min. The absorption of the so-called 'soluble tannins' was then measured at 550 nm. As shown in Terrill *et al.* (1992), a relatively large fraction of tannins is bound to proteins. This fraction was determined by a further extraction in which the solid residue was heated for 45 min at 95 °C with 6 mL of a sodium dodecyl sulphate (SDS) solution (10 g of SDS, 50 mL mercaptoethanol, made up with distilled water to 1 L) and then centrifuged. This procedure was repeated and the combined supernatants were brought to a volume of 20 mL using the SDS solution described above. A 1-mL aliquot of the resulting solution was heated together with 6 mL of freshly prepared BuOH–HCl solution at 95 °C for 75 min. The absorption was measured at 550 nm (protein-bound tannins). In order to relate optical densities to tannin concentrations, reference curves of extracted and purified tannins of *O. viciifolia* dissolved in either water (for the soluble tannins) or in SDS solution (for the protein-bound tannins) were used (Terrill *et al.*, 1992). All concentrations of CTs reported in this article refer to the sum of soluble and protein-bound CTs.

Non-structural carbohydrates (NSCs), defined here as the sum of glucose, fructose, sucrose and starch, were analysed in unscathed and in wounded leaflets as described in Wong (1990) and Körner *et al.* (1995). To do this, 10 mg of plant powder were extracted in boiling water and centrifuged, and a sample of the aqueous extract was treated with hexose isomerase and invertase to convert the fructose and sucrose into glucose. This, in turn, was converted enzymatically to glucose 6-phospate, and quantified photometrically by use of glucose 6-phosphate dehydrogenase plus NAD (glucose diagnostic kit from Sigma Diagnostics G3293, St Louis, MO, USA). The remainder of the water extract (including sugar and starch) was incubated with a dialysed crude fungal amylase ('Clarase' from *Aspergillus oryzae*; Enzyme Solutions Pty Ltd, Croydon South, Victoria, Australia) for 15 h at 40 °C to break down starch to glucose. Thereafter, total glucose was determined as described above.

Phosphorus and nitrogen concentrations in the leaflets were determined using the pooled material for each block and level of phosphorus availability. Phosphorus was analysed after wet digestion of the plant powder with concentrated H_2SO_4 and H_2O_2 (Jones and Case, 1990) by colorimetric quantification of the blue molybdophosphate complex (Murphy and Riley, 1962). Nitrogen was quantified using a CHN-analyser (Euro EA 3000; HEKAtech GmbH, Weinberg, Germany).

Statistical analysis

Plant height, plant biomass, NSC data, and local and systemic CT concentrations were analysed in split-plot models. Each model included coefficients for the blocks, for phosphorus availability (main plot factor) and for the wounding and elicitor treatments (sub-plot factor). Additionally, the model contained coefficients for the interaction between the wounding and elicitor treatments and phosphorus availability (interaction Ph × T). To avoid a violation of the assumption of normally distributed residuals, plant biomass was log-transformed prior to the analysis. All other data were used untransformed.

In addition to the global *F*-tests, more specific hypotheses were addressed by formulating linear contrasts between marginal means (i.e. between certain levels of phosphorus availability or between certain wounding and elicitor treatments). Specific comparisons with regard to phosphorus availability included contrasts between the following treatments: (*a*) the lowest and the highest level of phosphorus availability; (*b*) the lowest and the third (and for growth optimal) level of phosphorus availability; and (*c*) the third and the fourth level of phosphorus availability. Specific comparisons with regard to the wounding and elicitor treatments included contrasts between: (*a*) unscathed and wounded plants (effect of the wound alone) and (*b*–*d*) wounded plants and each elicitor treatment (test of the efficacy of the fungal, bacterial and insectan elicitor, respectively). Subsequently, each contrast was tested (*t*-test) using the estimated error variance suggested by the ANOVA model and the corresponding number of degrees of freedom. The family-wise error rate ($\alpha = 0{\cdot}05$) was controlled for the tests concerning phosphorus availability and the tests concerning the effect of the wound or the elicitors, using Bonferroni–Holm *P*-value corrections.

For the elemental analyses, plant samples were pooled within each block and the level of phosphorus availability and data were analysed in a two-way ANOVA with the factors block and phosphorus availability. Pairwise comparisons between the different levels of phosphorus availability were subsequently made using Tukey's honest significant difference. All data analyses were performed in R (version 2·5·1; www.r-project.org).

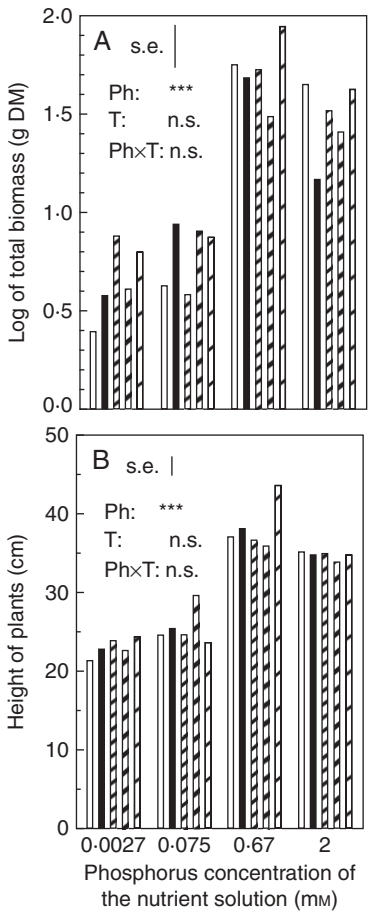

FIG. 2. (A) Total biomass (log-transformed) and (B) mean plant height of unscathed (white), wounded (black), and elicitor-treated plants (hatched columns, from left to right: bacteria, fungi and insects) at the end of the experiment ($n = 6$). For statistical details see Table 1.

RESULTS

Growth responses

The average total biomass of plants immediately before they were treated with one of four nutrient solutions was 512 ± 31 g d. wt. Thereafter, mean relative growth rates (ranked in order of increasing phosphorus availability) were 8·0, 9·7, 28·0 and 21·1 % d^{-1}. By the end of the experiment, the plants in the four solutions differed considerably in size (Fig. 2), with average heights of 23·0, 25·6, 38·3 and 34·7 cm, respectively (ANOVA: $P < 0{\cdot}001$), and average dry weights of 2·15, 2·50, 6·24 and 5·05 g (ANOVA: $P < 0{\cdot}001$). There were no significant differences in height or biomass between unscathed and wounded plants, and no differences among the various elicitor treatments (Table 1). Thus, growth depended only on the phosphorus concentration in the nutrient solution, with the third highest level being the most favourable.

Phosphorus and nitrogen contents of leaflets

The phosphorus content of leaf tissue increased with increasing phosphorus concentration of the nutrient solution (ANOVA: $P < 0{\cdot}001$; Fig. 3). Plants grown at the lowest level of phosphorus availability and, to a lesser extent, those at the second lowest showed typical symptoms of phosphorus deficiency such as purple margins to leaflets and necrotic areas. The phosphorus content of leaflets increased strongly and significantly from the second to the third level of phosphorus availability, and also from the third to the highest level of phosphorus availability. The phosphorus content at the highest level of phosphorus availability ($4{\cdot}66 \pm 0{\cdot}30$ mg P g^{-1} d. wt) was more than twice that at the lowest ($2{\cdot}19 \pm 0{\cdot}18$ mg P g^{-1} d. wt). The nitrogen content of leaflets increased only slightly with increasing phosphorus availability from $43{\cdot}31 \pm 1{\cdot}05$ mg N g^{-1} d. wt at the lowest level to $49{\cdot}27 \pm 2{\cdot}61$ mg N g^{-1} at the highest (Fig. 3; ANOVA: $P < 0{\cdot}05$). As a result, the nitrogen : phosphorus (N/P) ratio of leaflets ranged considerably, from 19·8 at the lowest phosphorus availability to 10·6 at the highest.

Non-structural carbohydrates

Except for a small necrotic area around each wound, wounded leaflets remained green and apparently active. Nevertheless, to assess whether wounding had affected carbon assimilation, the content of NSCs (i.e. the sum of glucose, fructose, sucrose and starch) was measured in unscathed and in wounded leaflets (Table 2). Contrary to what had been expected, carbohydrate levels were higher in wounded leaflets than in unscathed ones at all levels of

TABLE 1. *Summary of results*

		Height		Biomass		CT_L		CT'_L		CT_S	
	d.f.	(cm)	Δ %	log (g d. wt)	Δ %	(mg g^{-1} d. wt)	Δ %	(mg g^{-1} d. wt-NSC)	Δ %	(mg g^{-1} d. wt)	Δ %
Main plot:											
Block	5	n.s.		n.s.		n.s.		n.s.		n.s.	
Phosphorus	3	***		***		*		*		*	
Residuals	15										
Sub-plot:											
Wounding and elicitors (T†)	4	n.s.		n.s.		***		***		n.s.	
Phosphorus × T	12	n.s.		n.s.		n.s.		n.s.		n.s.	
Residuals	80										
Contrasts:											
P1 → P4	15	***	**50·9**	***	**134·2**	*	**− 17·3**	**	**−17·6**	*	**−17·6**
P1 → P3	15	***	**66·4**	***	**189·6**	n.s.	−11·4	*	**−12·4**	n.s.	−11·2
P3 → P4	15	*	**−9·3**	*	**−19·1**	n.s.	−6·6	n.s.	−5·9	n.s.	4·0
Unscathed → wounded (w)	80	n.s.	2·4	n.s.	−8·0	*	**−17·3**	*	**−12·3**	n.s.	−7·3
Wounded → w + bacteria	80	n.s.	−0·6	n.s.	14·9	*	**19·6**	**	**19·6**	n.s.	−2·1
Wounded → w + fungi	80	n.s.	1·0	n.s.	3·5	*	**15·9**	*	**15·8**	n.s.	5·3
Wounded → w + insect	80	n.s.	4·7	n.s.	25·1	***	**29·2**	***	**31·0**	n.s.	0·6

Levels of significance for the global H_0 hypotheses (F-tests; split-plot ANOVAs) and for the more specific linear contrasts including the relative change of the response variable (Δ %). The response variables are the plant height (cm), the log-transformed total biomass (g d. wt), the local CT concentration on a dry weight basis CT_L (mg g^{-1} d. wt), the local CT concentration on a non-structural carbohydrate-free dry weight basis CT'_L (mg g^{-1} d. wt-NSC) and the systemic CT concentration CT_S (mg g^{-1} d. wt).

For the linear contrasts t-tests have been used; the number of degrees of freedom (d.f.) corresponds to the degrees of freedom available for the estimation of the respective error variance in the ANOVA. The P-values of the linear contrasts were adjusted according to the Bonferroni–Holm method: the first three and the last four contrasts share $\alpha = 0{\cdot}05$ each. Asterisks correspond to the P-values of the statistical tests: ***, $P < 0{\cdot}001$; **, $P < 0{\cdot}01$; *, $P < 0{\cdot}05$; n.s., not significant. Significant relative differences are in bold.

† The test of T refers to the global F-test whether there are differences with regard of the target variable between any of the five wounding or elicitor treatments including the controls.

phosphorus availability ($P < 0{\cdot}01$). Furthermore, the content of NSCs in unscathed leaves tended to decrease with increasing phosphorus availability, although this result was not significant.

Concentrations of condensed tannins

The relationship between phosphorus availability and CT concentrations in the absence of any wounding or elicitor treatment was estimated using the mean data for the unscathed control plants (in which no distinction can be made between local and systemic effects). The results show a strong and highly significant decline in tannin concentrations with increasing phosphorus availability ($94{\cdot}9 \pm 5{\cdot}0$, $84{\cdot}9 \pm 6{\cdot}3$, $75{\cdot}5 \pm 6{\cdot}0$ and $69{\cdot}0 \pm 3{\cdot}6$ mg g^{-1} d. wt, respectively; ANOVA: $P < 0{\cdot}001$).

Wounding and the application of elicitors affected tannin concentrations locally (i.e. in the damaged leaflets; ANOVA local: $P < 0{\cdot}001$) but not systemically (i.e. in leaflets of undamaged neighbouring leaves; ANOVA systemic: $P = 0{\cdot}964$; Fig. 4). Tannin concentrations in leaflets that were wounded but not treated with an elicitor were lower than those in leaflets of unscathed plants ($P < 0{\cdot}01$). However, tannin concentrations in leaflets that had also been treated with one of the elicitors were significantly higher than those in leaflets that had only been wounded ($P < 0{\cdot}05$, $P < 0{\cdot}05$ and $P < 0{\cdot}001$ for the fungal, the bacterial and the insect elicitor, respectively; Table 1). For plants treated with either the bacterial or the fungal elicitor, this difference was not large and the CT concentrations were similar to those of unscathed plants. The only elicitor that increased tannin concentrations above the level of unscathed leaves was saliva of *Spodoptera littoralis* larvae. There was no evidence of a stronger stimulation of tannin concentrations by the wounding or elicitor treatments at low than at high nutrient availability (no significant Ph × T interaction; Table 1). All of these results remain essentially the same whether the tannin concentration is expressed as a percentage of the leaflet dry weight (CT_L) or of the NSC-free dry weight (CT'_L; Table 1).

It is commonly supposed that, at moderate to high levels of nutrients, tannin synthesis is 'costly' because it diverts carbon resources from plant growth (growth–defence trade-off), whereas at low nutrient availability producing tannins is 'cheap' because the availability of carbon exceeds growth demands (nutrient limitation of growth). Based on this concept, it was expected that, as phosphorus availability increased, an increasingly negative correlation between plant biomass and tannin concentrations of unscathed plants would be found. However, Pearson regression coefficients for this relationship – ranked from low to high phosphorus availability – were (−0·39), 0·50, 0·32 and 0·49, and none of them was significant.

DISCUSSION

In this experiment, foliar CT concentrations of *Onobrychis viciifolia* were inducible by elicitors derived from either microbes or herbivores, suggesting that regulation of the tannin concentration of this plant species is responsive to

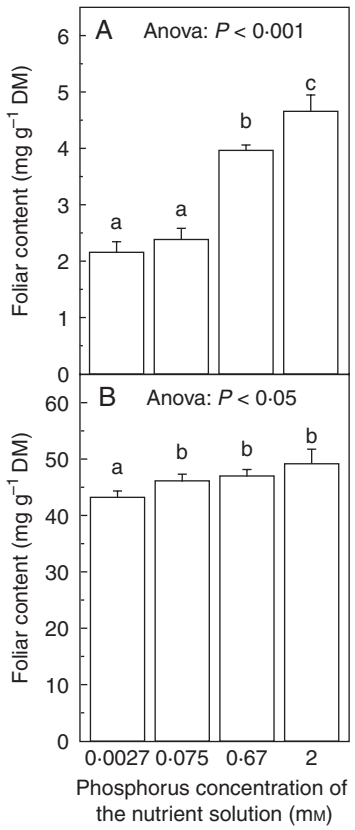

FIG. 3. (A) Foliar phosphorus and (B) nitrogen content at the end of the experiment. Columns represent block-wise pooled samples ($n = 6$). Columns that share a letter are not statistically different according to Tukey's honest significant difference.

TABLE 2. *Non-structural carbohydrates (mg g^{-1} d. wt) in unscathed and wounded leaflets at four levels of phosphorus concentration (mM) in the nutrient solution (mean ± s.e., $n = 6$)*

	P1 (0·0027 mM)	P2 (0·075 mM)	P3 (0·67 mM)	P4 (2 mM)
Unscathed	137·4 ± 19·1	104·5 ± 32·9	121·3 ± 17·2	80·0 ± 20·7
Wounded	168·0 ± 19·2	149·6 ± 22·4	152·7 ± 26·1	175·8 ± 47·3

the presence or absence of natural plant enemies. Declining CT concentrations with increasing nutrient availability were found, which in many previous experiments has been interpreted as evidence for a trade-off for resources between the production of secondary metabolites and plant growth at moderate to high nutrient levels. The experimental design with four levels of nutrient availability in combination with elicitors allowed some rarely tested but crucial sub-hypotheses of the underlying physiological model to be addressed (Herms and Mattson, 1992). As is discussed below, the present results seem incompatible with the physiological explanations provided by the growth-differentiation hypothesis, regarded by some authors as

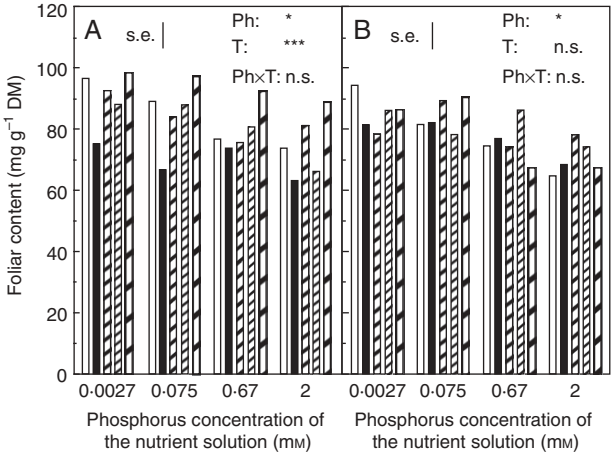

FIG. 4. (A) Local and (B) systemic tannin concentrations of unscathed (white), wounded (black), and elicitor-treated plants (hatched columns, from left to right: bacteria, fungi and insects) at the end of the experiment ($n = 6$). For statistical details see Table 1.

the most realistic of several plant defence hypotheses to be proposed (Koricheva, 2002; Stamp, 2003).

Onobrychis viciifolia responded to the experimental range of phosphorus availabilities with large differences in relative growth rate and chemical composition. The increasing phosphorus concentration in the nutrient solution was reflected in the phosphorus content of plant tissue and – due to its regulatory effect on nitrogen fixation (Almeida *et al.*, 1999) – also in the N content of the leaves. At the lowest level of phosphorus availability, plants showed typical deficiency symptoms, and growth was reduced compared with that of plants grown at higher phosphorus concentrations. At the second, third and fourth levels, phosphorus availability was sub-optimal, optimal and super-optimal for growth, respectively, as had been intended. The N/P ratio of plant tissue can be interpreted as an indicator of nutrient limitation and saturation (Tessier and Raynal, 2003): an N/P ratio of 13–17 suggests a balanced supply of both nitrogen and phosphorus, while lower and higher values indicate phosphorus and nitrogen limitation, respectively. Leaves in the lowest phosphorus treatment had an N/P ratio of 19·8, clearly indicating strong phosphorus limitation. Although the plants with the highest phosphorus availability had a low N/P ratio (10·6), this probably reflects excessive uptake of phosphorus rather than nitrogen limitation; indeed, the combination of vigorous growth and high nitrogen content of leaves (almost 50 mg N g^{-1} d. wt) suggests that the plants had plenty of nitrogen, most likely due to symbiotic N_2 fixation (Almeida *et al.*, 1999).

The lack of any difference in total biomass between unscathed and wounded plants, as well as the higher non-structural carbohydrate content of wounded than of unscathed leaflets, suggest that the wounding treatment itself did not hinder carbon assimilation. In a ^{13}C-labelling experiment with *Populus* saplings, Arnold and Schultz (2002) found that the import of assimilates into leaves increased after wounding, a process that might explain the

higher carbohydrate concentrations in the present study. These authors also found that, following damage, CT concentrations increased in developing sink leaves but not in fully developed source leaves. They concluded that the induction of tannin synthesis requires sink activity and the associated import of carbon from source leaves. Both the increased import of assimilates described by Arnold and Schultz (2002) and the higher carbohydrate status of wounded leaflets measured in the present experiment may represent an early condition of heightened alert following damage; subsequently, the presence of an enemy-derived elicitor may enable the plant to recognize the antagonist, thereby triggering conversion of these carbohydrates into the appropriate chemical defences.

Induction of secondary metabolism

The application of the regurgitant of *Spodoptera littoralis* caterpillars stimulated tannin concentrations in wounded leaflets of *Onobrychis viciifolia* beyond the level reached by any other treatment. Such a local increase in the concentration of CTs could have a physiological effect upon a herbivore, assuming that it continues to feed, but it is more likely to cause the insect to move and feed elsewhere (Bernays, 1981; Edwards and Wratten, 1983). The induction of CTs by *Spodoptera* saliva is consistent with several other studies; for example, Rossi *et al.* (2004) found that the protein-binding capacity of leaves of *Quercus myrtifolia* that had been mined or chewed by insects was 21–25 % higher than that of undamaged leaves. It is also in line with the repeated finding in various plants of a stimulated expression of central enzymes of the phenyl–propanoid pathway by the real or the simulated presence of herbivores or pathogens (Richard *et al.*, 2000; Peters and Constabel, 2002; Ralph *et al.*, 2006; Farag *et al.*, 2008).

The presence of fungal or bacterial elicitors increased tannin concentrations in the vicinity of the wound compared with leaflets that were wounded but not treated with an elicitor. This finding is consistent with a higher PAL activity and an increasing concentration of phenolic metabolites after the application of fungal or bacterial elicitors in the culture medium of different soybean cultivars (Groten and Barz, 2000). However, in the present experiment, the microbial elicitors were clearly less effective than caterpillar saliva and did not stimulate higher tannin concentrations than those in unscathed leaflets. The already relatively high constitutive level of CTs in *O. viciifolia* may help to protect wounds after cell rupture. In addition to CT, many (phenolic) substances have been identified in *Onobrychis viciifolia* extracts (e.g. kaempferol, quercetin, rutin, nicotiflorin and narcission; Marais *et al.*, 2000; Barrau *et al.*, 2005). Typically these substances occur in much lower concentrations than CT but some of them may play a role in plant defence against herbivores and/or pathogens.

No interaction between phosphorus availability and induced defence, no evidence of a growth–defence trade-off

Independently of the wounding or elicitor treatment, tannin concentrations decreased with increasing nutrient availability and growth rate, and with decreasing C/N ratios. However, no evidence was found for an interaction between the induction caused by any of the elicitors used in this study and the nutrient level to which the plant was exposed (Table 1). The lack of a stronger induction at low than at high nutrient availability, despite an apparent recognition of at least the insectan elicitor, seems incompatible with the common assumption that at low nutrient availability 'cheap' carbon would feed into the production of carbon-based secondary metabolites (Bryant *et al.*, 1983; Coley *et al.*, 1985; Herms and Mattson, 1992; Stamp, 2003). The fact that, depending upon phosphorus availability, biomass production varied by a factor of nearly three demonstrates the impressively large potential for carbon acquisition in excess of growth demands under some conditions. However, it appears that the relative availability of growth-limiting nutrients and carbon is not a good indicator for the degree of induction – perhaps because carbon is 'cheap' even at high nutrient availability (Craine *et al.*, 2003) or perhaps because the accumulation of CTs at low nutrient availability is somehow constrained. Negative correlations between growth rates and tannin concentrations *across* nutrient availabilities can result from passive dilution as well as from a trade-off between growth and defence, and should not automatically be interpreted as evidence for a growth defence trade-off (Koricheva, 1999; Häring *et al.*, 2007).

In this study, correlations between growth and tannin concentrations at any one level of phosphorus availability were always weak and never significant. With regard to their sign, the correlation coefficients were negative at the lowest level of phosphorus availability but positive at moderate to high nutrient availability – just the opposite of what the growth-differentiation balance hypothesis would predict. Thus, the present results contradict the idea of an 'inevitable' trade-off between the production of tannins and growth (Herms and Mattson, 1992; Craine *et al.*, 2003). Although carbohydrates and other storage-related compounds tend to accumulate when environmental conditions limit growth but not photosynthesis (Koricheva *et al.*, 1998; Hoch *et al.*, 2002; Hoch and Körner, 2003; Häring and Körner, 2004), this does not necessarily mean that the surplus carbon feeds into the pool of secondary metabolites (Koricheva *et al.*, 1998; Häring and Körner, 2004).

CONCLUSIONS

It was shown that concentrations of CTs in leaflets of *Onobrychis viciifolia* can be enhanced by the presence of substances originating from potential enemies. The saliva of the herbivorous insect *Spodoptera littoralis* stimulated local tannin concentrations more effectively than microbial elicitors. Overall, the present results are compatible with the hypothesis that foliar tannin concentrations decrease when enemies are absent and increase when they are present. However, the data suggest that trade-offs for resources between growth and defence are at least less general than predicted by the growth–differentiation balance hypothesis.

ACKNOWLEDGEMENTS

We are very grateful to Th. Boller, G. Felix and P. Salzer from the University of Basel who generously provided us with elicitors and to T. Turlings from the University of Neuchâtel from whom we had the *Spodoptera* caterpillars. We thank Ch. Staehelin from the University of Geneva and U. Merz from ETH Zurich for their help with the cultivation of *Rhizobia*. We thank G. Hoch from the University of Basel for the NSC-analysis and O. Huguenin-Elie, J. Leifeld and H. R. Bosshard from our own laboratories for their support with the analytical procedures. The project was financed by the Swiss Federal Office for Agriculture.

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